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	: He Lu	
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Title	: METHOD OF INHIBITING	
	: ANGIOGENESIS OR	
	: INVASION OR FORMATION	
	: OF METASTASES	

DECLARATION OF VÉRONIQUE TROCHON-JOSEPH MADE UNDER 37 CFR §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Véronique Trochon-Joseph, declare that I reside at 33, rue du Génie, 94400 Vitry sur seine, France. I attended the University of Rouen and received a Ph.D. degree in Parmacology Specialization in 1998. Since 2001, I have been employed by Bioalliancepharma Company as a Project Manager where I developed the anti-angiogenic and anti-invasive metargidin recombinant disintegrin peptide project. I have published 11 articles in international scientific papers.

I am one of the inventors named in the above-identified US Patent Application, that I am thoroughly familiar with the above referenced patent application and the subject matter described and claimed therein.

In an effort to demonstrate the fact that our original disclosure complies with 35 USC §112, we performed a number of experiments based on the original disclosure in our Specification. Those experiments and the results that we obtained are reported below.

Plasmid systems

Three different plasmid systems coding for the RDD (AMEP) gene were constructed : (i) the Tet-inducible pBi system, (ii) the constitutive pVAX plasmid and (iii) the pORT-RDD plasmid.

1) Tet-inducible pBi system

The RDD transgene was expressed under the control of a tetracycline-inducible Tet-On CMV promoter. The tetracycline system used is composed of three ampicillin resistant plasmids:

- The first plasmid (pBi-RDD) contains a CMV (cytomegalovirus) promoter with a tetracycline-responsive element (TRE) to drive the expression of the *AMEP* gene. The *RDD* gene under the control of the murine urokinase secretion signal was inserted between the $P_{MINCMV1}$ promoter and the β-globin polyA
- The second plasmid, pTet-On, expresses a strong transcriptional activator, rtTA.
- The third plasmid, pTet-tTS, encodes a powerful transcriptional silencer (tTS) that, in the absence of doxycycline, binds to the TRE sequence and blocks expression of the gene of interest.

2) the constitutive pVAX plasmid

pVAX1 (Invitrogen, ref V260-20) is a 3.0 kb plasmid vector designed for use in development of DNA vaccines. Features of the vector allow high-copy number replication in *E. coli* and high-level transient expression of the protein of interest in most mammalian cells. The vector contains the following elements:

- Human CMV promoter
- Bovine growth hormone (bGH) polyadenylation signal
- Kanamycine resistance gene for selection in *E. coli*

The RDD cassette, containing the human *AMEP* gene under the control of the murine urokinase secretion signal, was excised from the pBi-RDD and cloned in pVAX1 between the CMV and the BGH polyA, to generate the pVAX-RDD.

3) pORT-RDD plasmid

The ORT® technology (commercialized by the company named COBRA) employs a plasmid-mediated repressor titration to activate a host selectable marker, removing the requirement for a plasmid-borne marker gene. As an example, a strain of bacteria can be engineered that contains an essential gene such as dapD under transcriptional control of the lac operator/promoter (lacO/P). In the absence of an inducer such as lactose, this strain cannot grow due to the repression of dapD expression by the LacI repressor protein binding to lacO/P. Transformation with a high copy number plasmid containing the lac operator (lacO) effectively induces dapD expression by titrating LacI from the operator. Regulation of the essential gene ensures the growth of bacteria and maintenance of recombinant plasmids containing lacO and an origin of replication (Williams et al, Nucleic Acids Res. 1998 May 1;26(9):2120-4 ; Cranenburgh et al., Nucleic Acids Res. 2001 Mar 1;29(5):E26 ; Cranenburgh et al., J Mol Microbiol Biotechnol. 2004;7(4):197-203).

An expression plasmid, pORT-RDD, has been designed which encodes the RDD gene. More specifically, an expression cassette containing the RDD gene driven by human urokinase secretion signal was inserted into a pORT1aCMV, i.e. a ORT® plasmid containing a strong

eukaryotic constitutive promoter (CMV-intronA). The RDD expression cassette is thus under the control of the strong cytomegalovirus (CMV) promoter and placed upstream of the bovine growth hormone (bGH) polyadenylation signal.

Results with several cell models

1) Pre-established murine B16F10 melanoma model – Tet-inducible pBi system

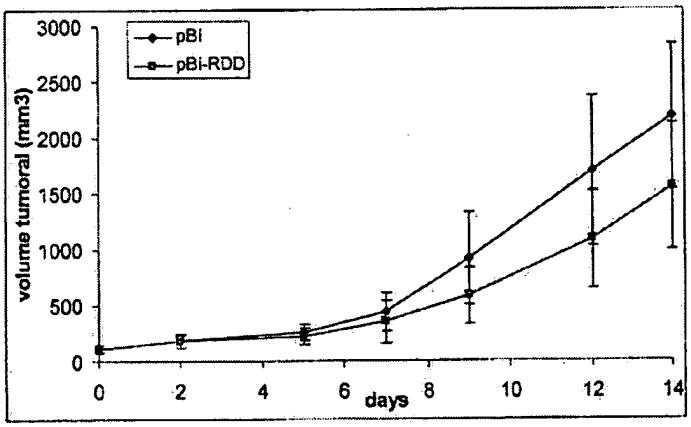
We injected 10^6 B16F10 murine melanoma cells in subcutaneous route onto the dorsa of C57Bl/6 mice. Tumors were intratumorally injected with 20µg of pBi control plasmid or pBi-RDD plasmid, mixed with 20µg of pTet-On and 10 µg of pTet-tTS plasmids (in 50µl NaCl 0,9%).

Conductive gel was applied on the tumor. The injection was immediately followed by electric pulses application by use of two stainless steel plate electrodes placed 5 mm apart on the tumor, according to the following protocol:

- HV = 1500 V/cm, 100 µs, 1 Hz, 1 pulse
- pause = 1 000 ms
- LV = 140 V/cm, 400 ms, 1 pulse

Day 0= treatment day.

Tumor size was monitored by measuring two perpendicular diameters with a digital caliper. Tumor volume was calculated according to the formula: $(\text{length} + \text{width}/2)^3 \times \pi/6$.



Summary: tumor volume in mm3

days	0	2	5	7	9	12	14
pBi	104.45	180.91	252.52	436.90	913.77	1692.89	2179.24
SD pBi	14.90	54.87	73.80	168.60	415.83	673.10	651.91
pBi-RDD	108.33	180.74	217.28	351.60	582.80	1083.70	1550.86
SD RDD	37.51	60.62	72.53	189.19	252.35	431.57	585.86
% inhibition		0.1	14.0	19.5	36.2	36.0	28.8
SigmaStat Student p=	0.764	0.995	0.295	0.301	0.045	0.03	0.05
					Mann&Whitney	0.055	0.092

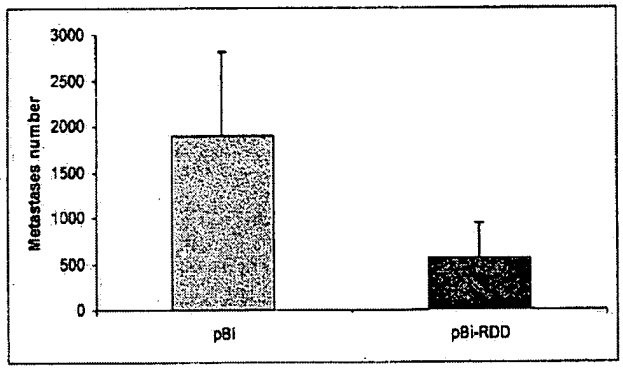
We observed a tumor growth inhibition in the murine melanoma B16F10 pre-established model. This inhibition (at least 30%) was significant from day 9 to day 14.

2) B16F10 metastatic model – Tet inducible pBi system

Day 0: Injection of 20µg of pBi control plasmid or pBi-RDD plasmid, mixed with 20µg of pTet-On and 10 µg of pTet-tTS plasmids (in 50µl NaCl 0,9%).
into both *Tibialis cranialis* muscles of C57Bl/6 mice, immediatcly followed by electrotransfer with the Cliniporator device

electrotransfer parameters : HV = 700 V/cm, 100µs, 1hz, 1 pulse
pause = 1000 ms
LV = 100 V/cm, 400 ms, 1 pulse

Day 3: Injection of 400 000 B16F10 cells (in 100µl) by IV route into the retro-orbital sinus.
Day 10 : Mice were sacrificed and lung metastatic loci were counted



	mean	SD	% inhib.	Student SigmaStat
pBi	1906.50	911.63		
pBi-RDD	581.75	378.01	70.5	0.008

We observed 70% of inhibition of the metastatic development with the pBi plasmid.

3) Pre-established human C9 melanoma model - constitutive pVAX plasmid

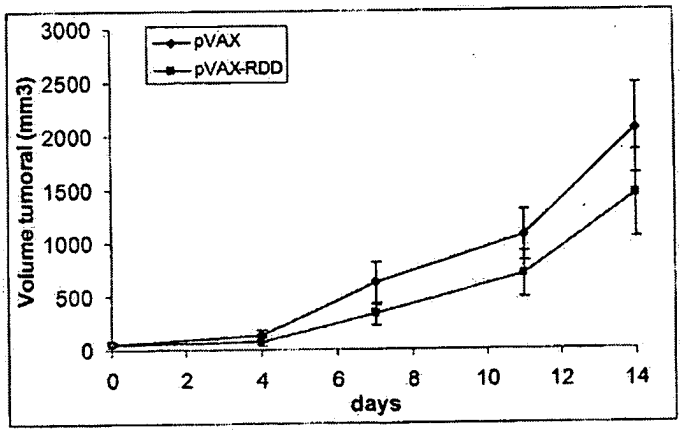
We injected $1x10^6$ C9 cells in subcutaneous route onto the dorsa of Swiss Nude mice. When mean tumor volume reached $50mm^3$, tumors were intratumorally injected with 50µg of pVAX control plasmid or 50µg of pVAX-RDD plasmid in 50µl.

Conductive gel was applied on the tumor. The injection was immediately followed by electric pulses application by use of two stainless steel plate electrodes placed 5 mm apart on the tumor, according to the following protocol:

- HV = 1500 V/cm, 100 µs, 1 Hz, 1 pulse
- pause = 1 000 ms
- LV = 140 V/cm, 400 ms, 1 pulse

Day 0= treatment day.

Tumor size was monitored by measuring two perpendicular diameters with a digital caliper. Tumor volume was calculated according to the formula: $(\text{length} + \text{width}/2)^3 \times \pi/6$.



Summary: tumor volume in mm3

days	0	4	7	11	14
pVAX	50.64	131.87	621.69	1066.99	2050.98
SD pVAX	18.17	50.53	195.15	235.19	425.19
pVAX-RDD	51.99	75.77	335.19	701.14	1443.22
SD pVAX-RDD	20.03	34.44	103.72	215.00	404.03
% inhibition		42.5	46.1	34.3	29.6
SigmaStat Student p=	0.900	0.029	0.004	0.010	0.018

We observed a tumor growth inhibition in the human melanoma C9 pre-established model. This inhibition (at least 30%) was significant from day 4 to day 14. The maximum of inhibition (46%) was obtained at day 7 post-treatment.

4) Study of antitumoral and antiangiogenic efficacy in subcutaneous B16F10 melanoma tumors - pORT-RDD intratumoral electrotransfer

The aim of this study was to investigate the efficacy of a single curative intratumoral electrotransfer of the new pORT-RDD plasmid in pre-established subcutaneous B16F10 tumors, compared to vehicle. The effect on tumor growth was assessed by tumor volume and tumor blood vessel monitoring by Doppler ultrasound sonography.

Mise en forme : Puces et numéros

- MATERIALS AND METHODS

Cell line and culture conditions

B16F10 melanoma cells were grown in Dulbecco's Modified Eagles'Medium + GlutaMAX supplemented with 10% heat inactivated foetal bovine serum, 1.5 g/l sodium bicarbonate and antibiotics (100 UI/ml penicillin and 100 µg/ml streptomycin; Invitrogen Gibco, ref 15140-122) at 37 °C in a humidified 5% CO2 atmosphere.

Animals

Female 6-8 week-old C57BL/6 mice were provided by Janvier (Le Genest-St-Isle, France). All animal experiments were performed according to ethical guidelines for animal experimentation (Directive n°86/609 CEE) and were approved by BioAlliance Pharma ethical committee.
Animals were acclimatized for at least 4 days before tumor cell implantation, in the area where the experiment took place. The animals were maintained in rooms under controlled conditions of temperature (21°C), photoperiod (12 h light/12 h dark) and air exchange (12 air renewals per hour). The humidity is kept between 30-70%.

Statistical tests

All statistical analyses were performed using SigmaStat 3.1 software. To compare two groups, the software ran the Student t test when normality and equal variance tests were successfully passed. If one of these tests failed, the software ran a Mann&Whitney rank sum test. For each analysis, the user was free to accept each of the statistical tests. A p value <0.05 was considered significant.

- EXPERIMENTAL STUDY DESIGN AND TREATMENT

Cell injection

B16F10 cells were subcultured 48 h before the day of injection.

Subconfluent B16F10 cells were rinsed with PBS, and then incubated with trypsin-EDTA solution (Invitrogen Gibco, ref 25300-096) until cells detached. Fresh medium was added, cells were centrifuged at 1200 rpm for 5 min, and resuspended in 25 ml of fresh medium. Cells were counted in 0.04% Trypan Blue for viability quantification. Cells were centrifuged and resuspended in the adequate volume of 0.9% NaCl (Versol, Laboratoire Aguettant, Lyon, France) in order to have 106 B16F10 cells in 100 µl.

100 µl of cells were injected by SC route on the right flank of mice. Flanks of mice were previously shaved with an electric razor the day before the cell injection.

Plasmid preparation

200µg of plasmid were injected in 50µl, leading to plasmid preparation at 4.0µg/µl by ethanol precipitation of pORT-RDD plasmid and resuspension in 10mM Tris, 1mM EDTA, 0.9% NaCl, pH7.5 sterile buffer.

Study design

C57BL/6 female mice bearing subcutaneous B16F10 tumors were assigned the following group numbers:

Table 4: Groups of treated mice

Group	Treatment	Dose (µg plasmid per tumor)	No of treatment	Route	No of animals per group
1	Vehicle		1	IT	5
2	pORT-RDD	50	1	IT	5

Plasmid electrotransfer (day 0)

The animals were anesthetized by intraperitoneal injection of 0.2 ml of a ketamine / xylazine mix containing: 0.5 ml xylazine 2% (Rompun®, Bayer), 2.0 ml ketamine 50 mg/ml (Ketalar, Panpharma, Fougères, France), 7.5 ml NaCl 0.9%.

The treatment consisted of a single intratumoral injection of 200 µg of plasmid in 50 µl of TE-NaCl buffer.

Conductive gel was applied on the tumor. The injection was immediately followed by electric pulses application by use of two stainless steel plate electrodes placed 5 mm apart on the tumor, with the Cliniporator® device according to the following protocol:

- HV = 1500 V/cm, 100 µs, 1 Hz, 1 pulse
- pause = 1 000 ms
- LV = 140 V/cm, 400 ms, 1 pulse.

Tumor volume monitoring and study of tumor growth

All the monitoring will be performed using an Aplio ultrasound device (Toshiba) equipped with two linear probes: 14 MHz probe for B mode and Power Doppler examination (better sensitivity and resolution), and 9 MHz probe for perfusion examination with contrast agent in VRI mode (Vascular Recognition Imaging).

Mice will be anesthetized by intraperitoneal injection of a ketamine/xylazine mix on day 0, and

by isoflurane inhalation on days 1, 2, 3, 4, and 7.

Tumor volume monitoring

Tumor volumes will be determined by measuring the width, length and depth of the tumor in B mode. The sonographic B mode allows representing organs morphology and texture according to a gray scale. On such B mode images, tumor dimensions can be measured and necrosis can be qualitatively appreciated by the extent of hypoechogenic areas.

Sonographic measurements will consist in finding the maximal transversal and longitudinal tumor sections on the scan using the 14 MHz linear probe. Transversal and longitudinal sections will be defined with respect to the mouse body. Length will be measured on the longitudinal section, width and depth on the transversal section. These measurements will be performed directly on the sonograph using calipers. Maximal transversal and longitudinal scans will be recorded and printed.

Tumor volumes will be calculated according to the formula:

Volume in mm3 = (length × width × depth)/2

Number of tumor blood vessels

Each tumor will be scanned both in transversal and longitudinal section in Doppler Power mode, with the 14MHz probe, in order to count the number of vessels into the tumor volume. The Doppler Power mode represents, in a color scale and in superposition with the B mode image, the ultrasound power backscattered by the circulating red blood cells in the vessels lumen. The video sequence corresponding to the tumor scan by successive probe displacements will be recorded allowing a 3D quantification on the whole tumor volume. These sequences will be post-reviewed by the operator in order to determine the number of intra-tumoral vessels. Color pixel clots will be considered as markers of an intra-tumoral vessel when these pixels will be repeatedly found in successive tumor sections showing a continuous follow-up of the blood flow. The mean number of vessels will be then calculated as the mean of vessels counted in transversal section and in longitudinal section.

• RESULTS

Mise en forme : Puces et numéros

Tumor volume monitoring

Tumor volume was significantly inhibited by 77.5% at day 4 and 89.2% at day 7 by pORT-RDD treatment compared to vehicle group (Student t test $p<0.001$ and $p=0.006$ respectively).

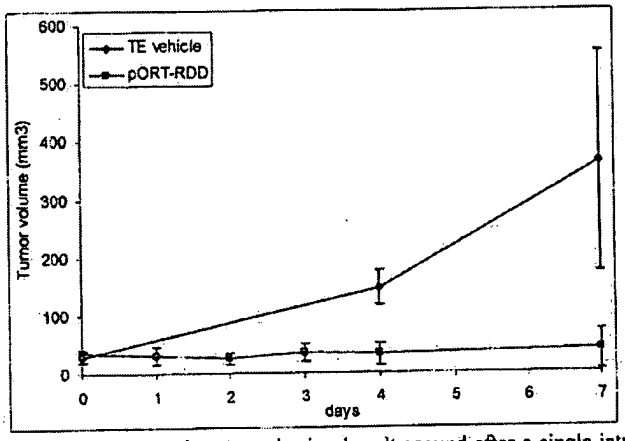


Figure 4: Tumor volume monitoring by ultrasound after a single intratumoral electrotransfer of 200µg of pORT-RDD plasmid.

Blood vessel monitoring

The number of tumor blood vessels was significantly inhibited by 70.1% at day 4 and 78.1% at day 7 by pORT-RDD treatment compared to vehicle group (Student *t* test $p<0.001$ and $p=0.001$ respectively).

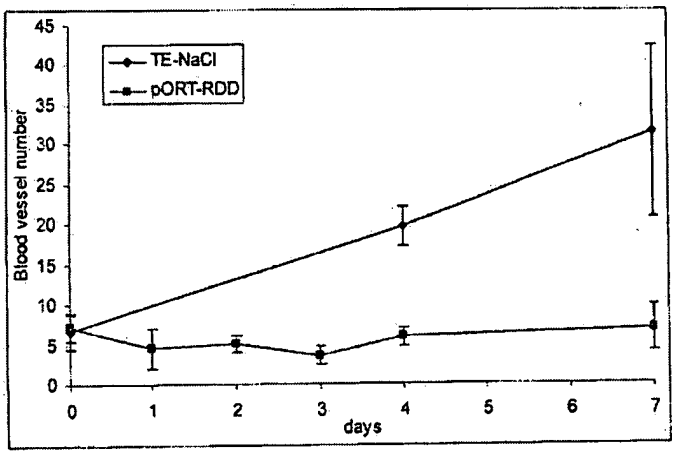


Figure 5: Tumor blood vessel monitoring by Doppler-ultrasound after a single intratumoral electrotransfer of 200µg of pORT-RDD plasmid.

Taken together, these results indicate that pORT-RDD intratumoral electrotransfer significantly inhibited the number of tumor blood vessels by up to 78%, in correlation with tumor growth inhibition. These results confirmed the dual activity of the disintegrin fragment on both endothelial and melanoma cells.

5) Evaluation of the efficiency of preventive intramuscular electrotransfer on B16F10 subcutaneous tumor growth - pORT-RDD plasmid

The aim of the present study is to determine the potential preventive effect of the pORT-RDD plasmid on B16F10 subcutaneous tumor growth when electrotransferred into mice muscle before inoculation of the tumoral cells.

The treatment consisted in the electrotransfer of 400µg of pORT-RDD plasmid in *Tibialis cranialis* muscles at day -1 (200 µg in 50 µl in each *Tibialis cranialis* muscle). Control animals received the vehicle under the same experimental conditions. Then, tumor cells were injected into the right flank of C57Bl/6J mice the day after electrotransfer treatment (day 0). Tumor development and then tumor volume were regularly monitored.

- EXPERIMENTAL STUDY DESIGN AND TREATMENT

Plasmid preparation

A total amount of 400µg of lyophilized pORT-RDD plasmid per mouse, in a volume of 100µl was injected (50 µl in each *Tibialis cranialis* muscle). Sterile freeze-dried vials of plasmid were reconstituted to 4mg/ml by addition of water for injection. The plasmid suspension was kept at 4°C during the injection time.

Study design

The study involved 30 C57BL/6J female mice.
Treatment allocation was decided at random and was as follows:

Group	Treatments	Dose per injection (µg in 50µl)	No of treatment	No of animals per group
1	Vehicle	none	2	15
2	pORT-RDD	200	2	15

Groups of treated mice

Before electroporation, the animals were anesthetized by intraperitoneal injection of 0.2 ml of a ketamine / xylazine mix.

The treatment consisted in an intramuscular injection of 200µg of plasmid in 50 µl of water for injection in each *Tibialis cranialis* muscle of mice, immediately followed by electroporation. A conductive gel was applied on the leg. The injection was immediately followed by electric pulses application by the use of two stainless steel plate electrodes placed 5 mm apart on the leg, with the Cliniporator device according to the protocol:

- HV = 700 V/cm (i.e. 350 V), 100 µs, 1 Hz, 1 pulse
- pause = 1 000 ms
- LV = 100 V/cm (i.e. 50 V), 400 ms, 1 pulse

Day-1 corresponded to the day of electrotransfer treatment.

The day before intramuscular electrotransfer, dorsa were shaved with an electric razor. The day of treatment, mice were identified by finger tattoo and legs were shaved.

Cell injection

B16F10 cells were injected the day after the electrotransfer treatment (day 0).

B16F10 cells were maintained in culture up to 8 passages before injection in mice. Cells were subcultured 48-72 h before the day of injection.

The day of cell inoculation, subconfluent B16F10 cells were rinsed with PBS, and then incubated at 37°C with tryspin-EDTA (Invitrogen Gibco, ref 25300-096) until cells detached. Fresh medium was added, cells were centrifuged at 1200 rpm for 5 min, and resuspended in 50 ml of fresh medium. Cells were counted in 0.04% Trypan Blue for viability quantification. Cells were centrifuged and resuspended in the adequate volume of 0.9% NaCl in order to have 10^6 B16F10 cells in 100 µl.

100 µl of cells were injected by subcutaneous route into the mice dorsa.

Animals monitoring and sacrifice

After B16F10 tumoral cell injection (day 0), mice were monitored regularly until tumors were palpable (about day 5-day 7). Then, tumor volumes were monitored regularly by measuring two perpendicular diameters (longest and largest diameters) with a digital calliper.

Tumor volume was calculated according to the formula: $(\text{length} + \text{width}/2)^3 \times \pi/6$. Inhibition of tumor growth was calculated as follows:

%Inhibition = 100 x [1 – (Tumor volume in treated group/Tumor volume in control group)].

- RESULTS

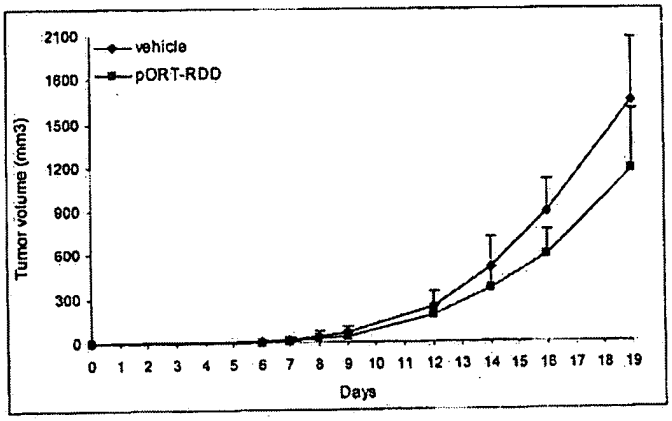


Figure 6: Tumor volume monitoring of B16F10 subcutaneous tumors treated by intramuscular electrotransfer of pORT-RDD plasmid and vehicle. Data represent the tumor volume (mean ± SD) for each group.

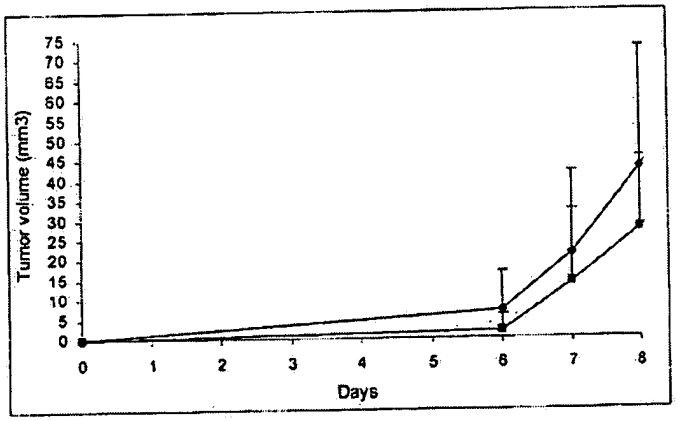


Figure 7: Tumor volume monitoring of B16F10 subcutaneous tumors treated by intramuscular electrotransfer of pORT-RDD plasmid and vehicle from day 0 (cells injection) to day 8. Data represent the tumor volume (mean ± SD) for each group.

Days	6	7	8	9	12	14	16	19
pORT-RDD vs vehicle	75.9%	35.1%	35.9%	34.5%	25.1%	28.7%	33.6%	28.7%
p test student	0.0733	0.3443	0.1414	0.1135	0.1032	0.0756	0.0024	0.0315

Percentage of tumor growth inhibition

Statistical analyses were performed using GraphPad Prism 4.0 software. A p value <0.05 was considered significant.

As shown in Figures 6 and 7 and in Table above, we observed that a 400 µg intramuscular pORT-RDD plasmid electrotransfer induced an inhibition of 75.9% of tumor growth at day 6 compared to the control vehicle group and about 25-35% from day 7 until the end of the study. This difference at day 6 between pORT-RDD treated group and control group can be explained by the delay of subcutaneous tumor implantation in treated group. Indeed, at this time of the experiment, fewer tumors (with smaller volume) were detected in mice.

We can noticed that the difference between treated group and control group was significant at day 16 (33.6%) and day 19 (28.7%) (p value<0.05).

Moreover, as shown in Table below, we observed that pORT-RDD plasmid treatment significantly slowed down B16F10 subcutaneous tumor growth. Indeed, the mean time to reach a tumor volume of 1000 mm^3 and 2000 mm^3 was decreased in treated group compared to control group.

	Time to reach tumor volume of 1000 mm^3	Time to reach tumor volume of 2000 mm^3
Vehicle group	16.4 days	19.7 days
p-AMEP group	18.2 days	21.1 days
p test student	0.0052	0.0158

Table 13: Mean time for growth of 1000 mm^3 and 2000 mm^3 subcutaneous B16F10 tumor in vehicle group and pORT-RDD plasmid treated group

Statistical analyses were performed using GraphPad Prism 4.0 software. A p value <0.05 was considered significant.

These results indicate that systemic AMEP, produced thanks to a muscle electrotransfer of the pORT-RDD, is able to inhibit B16F10 tumor growth implantation.
A repeated muscle electrotransfer treatment at 7 day interval may be able to increase this systemic protective effect.

The undersigned declares that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and thus such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:05/01/2009

Véronique Trochon-Joseph, Co-inventor